(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 February 2001 (08.02.2001)

PCT

(10) International Publication Number
WO 01/09175 A2

(51) International Patent Classification[7]: C07K 14/00

(21) International Application Number: PCT/GB00/02968

(22) International Filing Date: 31 July 2000 (31.07.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9918156.2 2 August 1999 (02.08.1999) GB

(71) Applicant *(for all designated States except US)*: **ZENECA LIMITED** [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **POSTHUMA, Geertruida, Afina** [NL/NL]; DLO Institute voor Dierhouderij en Diergezondheid, Postbus 65, NL-8200 AB Lelystad (NL). **SCHAAPER, Wilhelmus, Martinus, Maria** [NL/NL]; DLO Institute voor Dierhouderij en Diergezonheid, Postbus 65, NL-8200 AB Lelystad (NL). **SIJTSMA, Lolke** [NL/NL]; DLO Institute voor Agrotechnologisch Onderzoek, Bornesesteeg 59, P.O. Box 17, NL-6700 AA Wageningen (NL). **VAN AMERONGEN, Aart** [NL/NL]; DLO Institute voor Agrotechnologisch Onderzoek, Bornesesteeg 59, P.O. Box 17, NL-6700 AA Wageningen (NL). **FANT, Franky** [BE/BE]; University of Ghent, 25, Sintpietersnieuwstraat, B-9000 Ghent (BE). **BORREMANS, Frans, Alois, Mekania** [BE/BE]; University of Ghent, 25, Sintpietersnieuwstraat, B-9000 Ghent (BE). **REES, Sarah, Bronwen** [GB/GB]; Jealott's Hill Research Station, Bracknell, Berkshire RG42 6EY (GB). **OSBORN, Rupert, William** [GB/GB]; Plant Biotech Limited, Norwich Research Park, Colney Lane, Norwich NR4 7UH (GB).

(74) Agents: **GAAL, Jozsef, Christopher** et al.; Zeneca Agrochemicals, Intellectual Property Department, Jealott's Hill International Research Centre, P.O. Box 3538, Bracknell RG42 6YA (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/09175 A2

(54) Title: SYNTHETIC PEPTIDES

(57) Abstract: A modified cysteine-containing antimicrobial peptide derived from a plant defensin said modification comprising (a) introducing one or more cysteine residues and/or (b) replacing or altering one or more, cysteine residues to block their ability to form disulphide bridges. The modified peptides can have improved antimicrobial activity.

SYNTHETIC PEPTIDES

This invention relates to antimicrobial peptides, processes for their manufacture and use and DNA sequences encoding them.

Plant defensins are a class of small proteins displaying antimicrobial activity [Broekaert et al, Plant Physiol, 108:1353-1358 (1995), Crit. Rev. Plant Sci (1997)]. The first two antifungal proteins belonging to this class (Rs-AFP1 and Rs-AFP2) were isolated from radish seed (*Raphanus sativus*) [Terras et al J. Biol. Chem. 267:15301-15309(1992)]. Since then, many related proteins have been isolated from many plant species [Broekaert et al, Plant Physiol. 108:1353-1358 (1995), Osborn et al FEBS Lett. 368:257-262.(1995)]. A common structural motif in the family of plant defensins is an alpha-helix and three antiparallel beta-strands stabilized by highly conserved cysteine bridges. Other conserved amino acids include Gly-13, Gly-34, an aromatic residue at position 11, and Glu-29 [Broekaert et al Plant Physiol. 108:1353-1358 (1995). Plant defensins cause reduced hyphal elongation with (e.g. Rs-AFPs) or without (e.g. Ah-AMP1, from *Aesculus hippocastanum* seed [Osborn et al. FEBS Lett. 368:257-262 (1995)]) increase of hyphal branching. A third subclass of plant defensins inhibit alpha-amylases. Although the mechanism of the fungal growth inhibition is unknown, it was found that plant defensins cause an increased Ca^{2+} influx when added to *Neurospora crassa* hyphae [Thevissen et al. J. Biol. Chem. 271: 15018-15025. (1996)].

Rs-AFPs are antifungal proteins isolated from radish (*Raphanus sativus*) seed or leaves, which consist of 50 or 51 amino acids and which belong to the plant defensin family of proteins. Four highly homologous Rs-AFPs have been isolated (Rs-AFP1 to 4). The structure of Rs-AFP1 consists of three beta-strands and an alpha-helix and is stabilized by four cystine bridges [Fant *et al.* J. Mol. Biol., 279: 257-270 (1998)]. The isolation from biological sources yields only small amounts of material and chemical synthesis of the bioactive proteins is complex. Therefore, small peptides deduced from the native sequence, still having biological activity, are not only important tools to study structure-function relationships but also a commercially interesting target.

Cysteines, conserved in the native protein, are essential for the secondary structure of the protein. It has been described previously (WO 97/21815) that in certain cases,

where all cysteines in the peptide are substituted, activity is retained, although generally reduced.

the cysteines in the 19-residue peptides derived from the beta-2-beta-3 loop can be substituted by alpha-aminobutyric acid (Abu) and retain activity, sometimes even gaining potency. Analogous 19-mer peptides, forced to adopt a hairpin structure by introduction of one or two disulfide bridges were also found to possess high antifungal activity.

In a first aspect the invention therefore provides a modified cysteine-containing antimicrobial peptide derived from a plant defensin said modification comprising (a) introducing one or more cysteine residues and/or (b) replacing or altering one or more cysteine residues to block their ability to form disulphide bridges.

As used herein, the expression "modified cysteine-containing antimicrobial peptide" refers to a peptide which has at least one cysteine residue, but which is altered as described as compared to the corresponding naturally-occurring sequence from which it is derived. The peptide has antimicrobial activity, which is preferably enhanced in some way as compared to the corresponding naturally-occurring sequence from which it is derived.

The cysteine-containing antimicrobial peptides according to the invention are characterised in that the ability of the peptide to form disulphide bridges is altered when compared to the peptide or protein from which it is derived due to the addition of one or more cysteines to the peptide and/or to the alteration of one or more, and preferably not all, naturally occurring cysteine residues within said peptide wherein said alteration comprises blocking or altering the cysteine residue such that it is not capable of forming a disulphide bridge.

The addition of one or more cysteines to the peptide and/or the alteration of one or more, but preferably not all, naturally occurring cysteine residues within said peptide leads to the disruption of the natural disuphide bridge formation of the peptide by the formation of new non-natural disulphide bridges and/or by the removal of some of the existing di-sulphide bridges in the native peptide or protein from which the peptide is derived.

As used herein the term non-natural disulphide bridge denotes a disulphide bridge which does not naturally occur in the native peptide or protein from which the peptide is derived. The peptide may be derived from a larger peptide.

As used herein the term "plant defensin" is used to denote those proteins having antimicrobial activity and also having the following characteristic structural features: a cysteine residue at positions 4, 15, 21, 25, 36, 45, 47 and 51; disulphide bridge formation between the cysteines at positions 4 and 51, 15 and 36, 21 and 45 and 25 and 47; an aromatic amino acid residue 4 amino acids upstream from the cysteine at position 15, a glycine residue 2 amino acids upstream from the cysteine at position 15, a glutamic acid residue 7 amino acids upstream from the cysteine at position 36, and a glycine residue 2 amino acids upstream of the cysteine at position 36 wherein the positions of the cysteine residues are defined relative to the Rs-AFP1 sequence, as well as homologues, active variants and derivatives thereof. In some plant defensins the segments between positions 1 and 3, 5 and 14, 26 and 35 and 37 and 44 may vary in length by one to three amino acids but this does not affect the overall characteristic cysteine motif described above. This characteristic structural feature of the plant defensins is as follows:

...C.....a.G.C.....C...C...E....G.C.....C.C...C (SEQ ID NO 1)

where a is an aromatic amino acid (F,W,Y), C represents cysteine, E represents glutamic acid

and G is glycine and unspecified amino acids or groups of amino acids are represented by stops.

For example, the amino acid sequence of the plant defensin Rs-AFP2 is shown as SEQ ID NO 2 and that of Rs-AFP1 is shown as SEQ ID NO 3.

QKLCQRPSGTWSGVCGNNNACKNQCIRLEKARHGSCNYVFPAHKCICYFPC
(SEQ ID NO 2)

QKLCERPSGTWSGVCGNNNACKNQCINLEKARHGSCNYVFPAHKCICYFPC
(SEQ ID NO 3)

The expression "homologues" as used herein refers to any peptide which has some amino acids in common with the given sequence. Suitably at least 60% of the

amino acids will be similar, more suitably at least 70%, preferably at least 80%, more preferably at least 90% and most preferably at least 95%, 96%, 97%, 98% of amino acids will be similar to the corresponding amino acid in the given sequence.

As used herein the term "similar" is used to denote sequences which when aligned have similar (identical or conservatively replaced) amino acids in like positions or regions, where identical or conservatively replaced amino acids are those which do not alter the activity or function of the protein as compared to the starting protein. For example, two amino acid sequences with at least 85% similarity to each other have at least 85% similar (identical or conservatively replaced) amino acid residues in a like position when aligned optimally allowing for up to 3 gaps, with the *proviso* that in respect of the gaps a total of not more than 15 amino acid residues is affected. The degree of similarity may be determined using methods well known in the art (see, for example, Wilbur, W.J. and Lipman, D.J. "Rapid Similarity Searches of Nucleic Acid and Protein Data Banks." Proceedings of the National Academy of Sciences USA 80, 726-730 (1983) and Myers E.and Miller W. "Optimal Alignments in Linear Space". Comput. Appl. Biosci. 4:11-17(1988)). One programme which may be used in determining the degree of similarity is the MegAlign Lipman-Pearson one pair method (using default parameters) which can be obtained from DNAstar Inc, 1228, Selfpark Street, Madison, Wisconsin, 53715, USA as part of the Lasergene system.

Amino acids which differ from the basic sequence may be conservatively or non-conservatively substituted. A conservative substitution is to be understood to mean that the amino acid is replaced with an amino acid with broadly similar chemical properties. In particular conservative substitutions may be made between amino acids with the following groups:

(i) Alanine, Serine, Glycine and Threonine;

(ii) Glutamic acid and Aspartic acid;

(iii) Arginine and Lysine;

(iv) Asparagine and Glutamine;

(v) Isoleucine, Leucine, Valine and Methionine;

(vi) Phenylalanine, Tyrosine and Tryptophan.

In general, more conservative than non-conservative substitutions will be possible without destroying the antimicrobial properties of the compounds. Suitable homologues may be determined by testing antimicrobial properties of the peptide using routine methods, for example as illustrated hereinafter.

The term "variant" as used herein (other than in the sequence listings) includes experimentally generated variants or members of a family of related naturally-occurring peptides as may be identified by molecular genetic techniques. Such techniques are described for example in US Patent No. 5,605,793, US Patent No. 5,811,238 and US Patent No 5,830,721, the content of which is incorporated herein by reference. In essence this technique involves expression of the parental gene in a microbial expression system such as *Escherichia coli*. The particular system selected must be validated and calibrated to ensure that biologically active peptides are expressed, which may be readily achieved using a *in vivo* bioassay. The gene, or preferably a collection of related genes from different species, may be subject to mutagenic polymerase chain reaction (PCR) as is known in the art. Fragmentation of the products and subsequent repair using PCR leads to a series of chimeric genes reconstructed from parental variants. These chimeras are then expressed in the microbial system which can be screened in the usual way to determine active mutants, which may then be isolated and sequenced. Reiteration of this molecular evolution DNA shuffling cycle may lead to progressive enhancement of the desired gene properties. The advantage of a technique of this nature is that it allows a wide range of different mutations, including multi-mutation block exchanges, to be produced and screened.

Other variants may be identified or defined using bioinformatics systems. An example of such a system is the FASTA method of W.R. Pearson and D.J. Lipman PNAS (1988) 85:2444-2488. This method provides a rapid and easy method for comparing protein sequences and detecting levels of similarity and is a standard tool, used by molecular biologists. Such similar sequences may be obtained from natural sources, through molecular evolution or by synthetic methods and comparisons made using this method to arrive at "opt scores" which are indicative of the level of similarity between the proteins.

Particular variants of the invention will comprise antimicrobial proteins with an amino acid sequence with a FASTA opt score (as defined in accordance with FASTA version 3.0t82 November 1, 1997) against any one of the sequences of the proteins in the antimicrobial composition of the invention described herein as follows. Variants of the invention will comprise antimicrobial proteins with an amino acid sequence with a FASTA opt score (as defined in accordance with FASTA version 3.0t82 November 1, 1997) of greater than or equal to 300 against Rs-AFP1 or 2.

The term "derivative" relates to antimicrobial proteins which have been modified for example by using known chemical or biological methods.

Preferably, the modified peptides of the inventions are naturally-occurring plant defensins which have been modified in accordance with the invention.

The modification of the defensin according to the invention by the introduction of one or more cysteine residues is particularly preferred. Further the modification of the defensin according to the invention by the introduction of one or more cysteine residues in combination with the modification of one or more cysteine residues to block their ability to form disulphide bridges is particularly preferred.

In this and all further aspects of the invention the cysteine containing antimicrobial peptide is preferably derived from a plant defensin selected from the group Rs-AFP1, Rs-AFP2, Rs-AFP3, Rs-AFP4, Br-AFP1, Br-AFP2, Bn-AFP1, Bn-AFP2, Sa-AFP1, Sa-AFP2 and At-AFP1 and Hs-AFP1, Ah-AMP1 and Dm-AMP1 which are fully described in Published International Patent Applications Nos. WO 93/05153 and WO 95/18229 the teachings of which are incorporated herein by reference, Aly-AFP and Alf-AFP which are fully described in Published International Patent Applications Nos. WO 97/37024 and WO 98/26083 the teachings of which are incorporated herein by reference. The cysteine containing antimicrobial peptide is more preferably derived from a plant defensin selected from the group Rs-AFP1 or Rs-AFP2, and is most preferably derived from Rs-AFP2. It is further preferred that the peptide is derived from the beta-2 strand/turn/beta-3 strand region of a plant defensin. The secondary structure elements of RsAFP1 have been described by Fant *et al*; [J. Mol. Biol. 279: 257-270 (1998)].

In a particularly preferred embodiment the invention provides a modified cysteine containing antimicrobial derived from the beta-2 strand/turn/beta-3 strand region of a

plant defensin said modification comprising (a) introducing one or more cysteine residues and/or (b) replacing or altering one or more cysteine residues to block their ability to form disulphide bridges.

The beta-2 strand/turn/beta-3 strand region of a plant defensin may be determined by analysis of the primary amino acid sequence information and generally is predicted to be located between the fourth and the eighth cysteine residue. For example in Rs-AFP1 and Rs-AFP2 this occurs between positions 21 to 51 of the sequence, and more precisely at positions 30 to 51 of the sequence.

In a preferred embodiment the invention provides a modified cysteine containing antimicrobial peptide derived from positions 21 to 51 of Rs-AFP1 or Rs-AFP2 said modification comprising (a) introducing one or more cysteine residues and/or (b) replacing or altering or one or more cysteine residues to block their ability to form disulphide bridges.

In a further preferred embodiment the invention provides a modified cysteine containing antimicrobial peptide derived from positions 30 to 51 of Rs-AFP1 or Rs-AFP2 said modification comprising (a) introducing one or more cysteine residues and/or (b) replacing or altering one or more cysteine residues to block their ability to form disulphide bridges.

The one or more cysteine residues may be added at any position within the peptide sequence. The presence of such cysteine residues allows the formation, under appropriate conditions, of non-natural disulphide bridges within the peptide such that the conformation of the peptide is altered leading to a more rigid and less flexible structure. This may also result in improved biological activity as can been seen in the examples herein.

The di-sulphide bridges may be inter- disulphide bridges i.e. between two different protein molecules or may be intra-disulphide bridges i.e. occurring within one peptide molecule. The formation of inter-disulphide bridges is particularly preferred. The formation of inter-molecular disulphide bridges may lead to multimerisation and the formation of dimers and is particularly preferred especially where the peptide is a hexa- or 15- mer peptide. The preferred position for the added cysteine residue(s) is determined by considering an NMR model of the peptide and choosing the location where the two ends

of the native loop come together. This is particularly suitable where the peptide is long enough to form a complete loop structure and this will typically be 17-, 18- ,19- 20 mers and longer peptides.

It will be apparent to a man skilled in the art that where the peptide has been altered such that it has only one cysteine residue available for disulphide bridge formation then the disulphide bridges which will be formed will be inter-molecular disulphide bridges and the peptide structures will be dimeric.

We have also found that the addition of non-naturally occurring cysteine residues can enhance the activity of peptides where one or more naturally occurring cysteine residues have been replaced by a side chain blocked cysteine or by a closely related amino acid such as an alpha aminobutyric acid residue and this is a preferred embodiment of the invention. However, in an alternative embodiment, cysteine residues may be altered to another naturally amino acid, either by site-directed mutation using known methods, or preferably, by simply synthesising a peptide of the desired altered structure. This may be preferable, for example where it is desired to express the peptide in a plant as discussed below.

In a preferred embodiment of the first aspect of the invention the one or more cysteines are added to the N-terminal and/or C-terminal ends of the peptide.

The peptides to which the cysteines are added or in which the cysteines are altered are preferably at least 4, 5 or 6 amino acids in length, preferably at least 6 amino acids in length, more preferably at least 10 amino acids in length and most preferably are at least 15, 17, 19, 20 amino acids or longer in length.

The cysteines which are added to the peptides may be in D- and/or L-configuration and it is preferred that one, two, three or four cysteines are added to the peptide according to the invention. One or more cysteine residues naturally occurring in the peptides according to the invention may be replaced by D-cysteines.

In a further aspect the invention provides a plant defensin or a peptide derivative thereof comprising one or more cysteine residues in the D-configuration.

Naturally occurring cysteine residues may be blocked by a blocking group such as iodoacetamide or replaced by an alpha aminobutyric acid group or by an alternative amino

acid such that the formation of naturally occurring di-sulphide bridges involving said amino acid residues is inhibited.

In a particularly preferred embodiment of all aspects of the invention the antimicrobial peptide comprises the residues HGS and/or HKY.

In a further aspect the invention provides a method of making antimicrobial peptides more rigid comprising modifying the peptide structure by (a) introducing one or more cysteine residues and/or (b) replacing or altering one or more cysteine residues to block their ability to form disulphide bridges.

In a preferred further aspect the invention provides a method of making antimicrobial peptides more rigid comprising modifying the peptide structure by (a) introducing one or more cysteine residues and/or (b) replacing or altering or one or more, but not all, cysteine residues to block their ability to form disulphide bridges.

As used herein the term 'rigid' is used to denote that the peptide is less flexible in solution and that certain natural conformations have been excluded by the addition or removal of disulphide bridge(s). Therefore, in general, the structure will contain a heightened level of either intra- or inter-molecular bridges, and the elimination of linear structures which cannot form inter-molecular bridges. Suitably, this has the effect of enhancing a desired activity of the peptide, such as the antimicrobial activity. The antimicrobial peptide is preferably derived from a plant defensin as described herein.

In a further aspect the invention provides a method of improving the antimicrobial activity of an antimicrobial peptide derived from a plant defensin said method comprising altering the natural disulphide bridge pattern of the peptide by (a) introducing one or more cysteine residues and/or (b) replacing or altering or one or more, but not all cysteine residues to block their ability to form disulphide bridges.

Antifungal proteins which show the characteristic structural features of the plant defensins include the proteins Rs-AFP1, Rs-AFP3, Rs-AFP4, Br-AFP1, Br-AFP2, Bn-AFP1, Bn-AFP2, Sa-AFP1, Sa-AFP2 and At-AFP1 and Hs-AFP1, Ah-AMP1 and Dm-AMP1 are fully described in Published International Patent Applications Nos. WO 93/05153 and WO 95/18229 the teachings of which are incorporated herein by reference. It is particularly preferred that the peptides of the invention are derived from proteins having substantially similar activity to Rs-AFP2 and Rs-AFP1. Peptides derived from

plant defensins are described in Published International Patent Applications No WO 97/21815 the teachings of which are incorporated herein by reference and such peptides described therein are particularly useful starting peptides for modification according to the invention said modification comprising introducing one or more additional cysteine residues and/or altering

one or more, but not all, cysteine residues to block the ability to form disulphide bonds.

Antimicrobial peptides according to the invention include especially peptides derived from the beta-2 strand/turn/beta-3 strand region of Rs-AFP2 and proteins, such as homologues and variants, having substantially similar activity.

Antimicrobial peptides derived from the regions defined herein of the Rs-AFP plant defensins exhibit antifungal activity. Such peptides may be easier to synthesise than the full length plant defensin while retaining antifungal activity. DNA sequences encoding the peptides may also be more suitable for transformation into biological hosts.

An antimicrobial peptide according to the invention may be manufactured from its known amino acid sequence using the appropriate techniques such as by chemical synthesis, or produced within a suitable organism (for example, a micro-organism or plant) by expression of recombinant DNA. The antimicrobial peptide is useful as a fungicide and may be used for agricultural or pharmaceutical or other applications. The antimicrobial peptide may be used in combination with one or more other antimicrobial peptides of the present invention.

Knowledge of its primary structure enables manufacture of the antimicrobial peptide, or parts thereof, by chemical synthesis. It will be appreciated by one skilled in the art that when the peptides contain a chemical blocking group as described herein, they require synthesis or modification by a chemical route. Where appropriate, it also enables production of DNA constructs encoding the antimicrobial peptide.

The invention further provides a DNA sequence encoding an antimicrobial peptide according to the invention. The DNA sequence may be predicted from the known amino acid sequence and DNA encoding the peptide may be manufactured using a standard nucleic acid synthesiser.

The DNA sequence encoding the antimicrobial peptide may be incorporated into a DNA construct or vector in combination with suitable regulatory sequences (promoter,

terminator, transit peptide, etc). For some applications, the DNA sequence encoding the antimicrobial peptide may be inserted within a coding region expressing another protein to form an antimicrobial fusion protein or may be used to replace a domain of a protein to give that protein antimicrobial activity. The DNA sequence may be placed under the control of a homologous or heterologous promoter which may be a constitutive or an inducible promoter (stimulated by, for example, environmental conditions, presence of a pathogen, presence of a chemical). The transit peptide may be homologous or heterologous to the antimicrobial protein and will be chosen to ensure secretion to the desired organelle or to the extracellular space. The transit peptide is preferably that naturally associated with the antimicrobial protein of interest. Such a DNA construct may be cloned or transformed into a biological system which allows expression of the encoded peptide or an active part of the peptide. Suitable biological systems include micro-organisms (for example, bacteria such as Escherichia coli, Pseudomonas and endophytes such as Clavibacter xyli subsp. cynodontis (Cxc); yeast; viruses; bacteriophages; etc), cultured cells (such as insect cells, mammalian cells) and plants. In some cases, the expressed peptide may subsequently be extracted and isolated for use.

The invention further provides the use of a peptide according to any of the preceding claims in the treatment or prevention of microbial infections, preferably fungal infections.

An antimicrobial peptide according to the invention is useful for combating microbial diseases in plants. The invention further provides a process of combating fungi whereby they are exposed to an antimicrobial peptide according to the invention. The antimicrobial peptide may be used in the form of a composition.

The antimicrobial peptide or protein may be used in the form of a composition, for example in combination with a suitable carrier or diluent. For example, for agricultural use, compositions of the invention may be in the form of either a dilute composition which is ready for immediate use, or a concentrated compositions which require dilution before use, usually with water. Liquid compositions may contain other conventional components such as surface-active agents, dispersants etc.

Solid compositions may be in the form of granules, or dusting powders wherein the active ingredient is mixed with a finely divided solid diluent, e.g. kaolin,

bentonite, kieselguhr, dolomite, calcium carbonate, talc, powdered magnesia, Fuller's earth and gypsum. They may also be in the form of dispersible powders or grains, comprising a wetting agent to facilitate the dispersion of the powder or grains in liquid. Solid compositions in the form of a powder may be applied as foliar dusts.

For pharmaceutical applications, the antimicrobial peptide (including any product derived from it) may be used as a fungicide to treat mammalian infections (for example, to combat yeasts such as Candida).

Pharmaceutical compositions of the invention may be in a form suitable for oral use (for example as tablets, lozenges, hard or soft capsules, aqueous or oily suspensions, emulsions, dispersible powders or granules, syrups or elixirs), for topical use (for example as creams, ointments, gels, or aqueous or oily solutions or suspensions), for administration by inhalation (for example as a finely divided powder or a liquid aerosol), for administration by insufflation (for example as a finely divided powder) or for parenteral administration (for example as a sterile aqueous or oily solution for intravenous, subcutaneous, intramuscular or intramuscular dosing or as a suppository for rectal dosing). For further information on Formulation the reader is referred to Chapter 25.2 in Volume 5 of Comprehensive Medicinal Chemistry (Corwin Hansch; Chairman of Editorial Board), Pergamon Press 1990.

An antimicrobial peptide (including any product derived from it) according to the invention may also be used as a preservative (for example, as a food or cosmetics additive).

For agricultural applications, the antimicrobial peptide may be used to improve the disease-resistance or disease-tolerance of crops either during the life of the plant or for post-harvest crop protection. Pathogens exposed to the peptides are inhibited. The antimicrobial peptide may eradicate a pathogen already established on the plant or may protect the plant from future pathogen attack. The eradicant effect of the peptide is particularly advantageous.

Exposure of a plant pathogen to an antimicrobial peptide may be achieved in various ways, for example:

(a) The isolated peptide may be applied to plant parts or to the soil or other growth medium surrounding the roots of the plants or to the seed of the plant before it is sown using standard agricultural techniques (such as spraying).

The peptide may have been extracted from plant tissue or chemically synthesised or extracted from micro-organisms genetically modified to express the peptide. The peptide may be applied to plants or to the plant growth medium in the form of a composition comprising the peptide in admixture with a solid or liquid diluent and optionally various adjuvants such as surface-active agents. Solid compositions may be in the form of dispersible powders, granules, or grains.

(b) A composition comprising a micro-organism genetically modified to express the antimicrobial peptide may be applied to a plant or the soil in which a plant grows.

(c) An endophyte genetically modified to express the antimicrobial peptide may be introduced into the plant tissue (for example, via a seed treatment process).

An endophyte is defined as a micro-organism having the ability to enter into non-pathogenic endosymbiotic relationships with a plant host. A method of endophyte-enhanced protection of plants has been described in a series of patent applications by Crop Genetics International Corporation (for example, International Application Publication Number WO90/13224, European Patent Publication Number EP-125468-B1, International Application Publication Number WO91/10363, International Application Publication Number WO87/03303). The endophyte may be genetically modified to produce agricultural chemicals. International Patent Application Publication Number WO94/16076 (ZENECA Limited) describes the use of endophytes which have been genetically modified to express a plant-derived antifungal peptide.

(d) DNA encoding an antimicrobial peptide may be introduced into the plant genome so that the peptide is expressed within the plant body (the DNA may be cDNA, genomic DNA or DNA manufactured using a standard nucleic acid synthesiser).

Plant cells may be transformed with recombinant DNA constructs according to a variety of known methods (Agrobacterium Ti plasmids, electroporation, microinjection, microprojectile gun, etc). The invention extends to a plant cell transformed with a DNA construct according to the invention. The transformed cells may then in suitable cases be

regenerated into whole plants in which the new nuclear material is stably incorporated into the genome. Both transformed monocotyledonous and dicotyledonous plants may be obtained in this way, although the latter are usually more easy to regenerate. Some of the progeny of these primary transformants will inherit the recombinant DNA encoding the antimicrobial peptide(s).

We have found that the antimicrobial peptides according to the invention show activity and are particularly useful against a broad spectrum of plant pathogenic fungi. The peptides of the invention may also be useful in combating bacterial infections.

The invention further provides a plant having improved resistance to a microbial pathogen especially a fungal pathogen and containing recombinant DNA which expresses an antimicrobial peptide according to the invention. Such a plant may be used as a parent in standard plant breeding crosses to develop hybrids and lines having improved fungal resistance.

Recombinant DNA is DNA, preferably heterologous, which has been introduced into the plant or its ancestors by transformation. The recombinant DNA encodes an antimicrobial peptide expressed for delivery to a site of pathogen attack (such as the leaves). The DNA may encode an active subunit of an antimicrobial peptide.

A pathogen may be any fungus growing on, in or near the plant. In this context, improved resistance is defined as enhanced tolerance to a fungal pathogen when compared to a wild-type plant. Resistance may vary from a slight increase in tolerance to the effects of the pathogen (where the pathogen in partially inhibited) to total resistance so that the plant is unaffected by the presence of pathogen (where the pathogen is severely inhibited or killed). An increased level of resistance against a particular pathogen or resistance against a wider spectrum of pathogens may both constitute an improvement in resistance. Transgenic plants (or plants derived therefrom) showing improved resistance are selected following plant transformation or subsequent crossing.

Where the antimicrobial peptide is expressed within a transgenic plant or its progeny, the fungus is exposed to the peptide at the site of pathogen attack on the plant. In particular, by use of appropriate gene regulatory sequences, the peptide may be produced in vivo when and where it will be most effective. For example, the peptide may

be produced within parts of the plant where it is not normally expressed in quantity but where disease resistance is important (such as in the leaves).

Examples of genetically modified plants which may be produced include field crops, cereals, fruit and vegetables such as: canola, sunflower, tobacco, sugarbeet, cotton, soya, maize, wheat, barley, rice, sorghum, tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, melons, potatoes, carrot, lettuce, cabbage, onion.

The antifungal peptides MBG03 CRHGSBNYVFPAHKBIBYC (SEQ ID NO 21), MBG04 CRHGScNYVFPAHKcIBYC (SEQ ID NO 22) , MBN06 CRHGSCNYVFPAHKCIBYC (SEQ ID NO 25), MCH32 and MCH32x HKBIBYC (SEQ ID NO 4), MCH33 and MCH33x CHKBIBY (SEQ ID NO 5), MCH37 and MCH37x HKCIBY (SEQ ID NO 7), MCH38 and MCH38x HKBICY (SEQ ID NO 8), MCI28 and MCI28x CHKBIBYC (SEQ ID NO 10), MCI10 and MCI10x CHGSBNYVFPAHKBIBC (SEQ ID NO 11), MCI11 and MCI11x CHGSBNYVFPAHKBIB (SEQ ID NO 12), MCI12 and MCI12x HGSBNYVFPAHKBIBC (SEQ ID NO 13), MCI15 and MCI15x HGSCNYVFPAHKBIB (SEQ ID NO 15), MCI16 and MCI16x HGSBNYVFPAHKCIB (SEQ ID NO 16) and MCI17 and MCI17x HGSBNYVFPAHKBIC (SEQ ID NO 17), described more fully herein are particularly preferred. In the above, lower case "c" represents D-cysteine.

Most especially preferred peptides according to the invention are MBG03 CRHGSBNYVFPAHKBIBYC (SEQ ID NO 21), MBG04 CRHGScNYVFPAHKcIBYC (SEQ ID NO 22), MBN06 CRHGSCNYVFPAHKCIBYC (SEQ ID NO 25) MCH32x HKBIBYC (SEQ ID NO 4), MCH38 and MCH38x HKBICY (SEQ ID NO 8), MCI12x HGSBNYVFPAHKBIBC (SEQ ID NO 13), MCI16 and MCI16x HGSBNYVFPAHKCIB (SEQ ID NO 16) and MCI17 and MCI17x HGSBNYVFPAHKBIC (SEQ ID NO 17).

The invention will now be described by way of example only, with reference to the following examples.

EXAMPLES

EXPERIMENTAL PROCEDURES

Materials. N-methylpyrrolidone (NMP), dimethylformamide (DMF), N-hydroxybenzotriazole (HOBt), 2-(1H-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluoro-phosphate (HBTU) and piperidine were peptide synthesis grade and obtained from Perkin Elmer/ABI (Warrington, UK). Acetonitrile (ACN) was gradient grade, diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), thioanisole (TA), phenol, and ethanedithiol (EDT) were synthesis grade and were obtained from Merck (Darmstadt, Germany). Before use, diethylether was purified over a column of activated basic aluminumoxide and DIEA was distilled twice over ninhydrin and potassiumhydroxide. Fmoc-amino acid derivatives and resin (4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin (Rink resin) were obtained from Saxon Biochemicals (Hannover, Germany).

Analytical HPLC. For analytical HPLC we used two Waters pumps model 510, a Waters gradient controller model 680, a Waters WISP 712 autoinjector, and a Waters 991 photodiode array detector. Products were analyzed in a linear gradient from water with 0.1% (v/v) TFA to 60% (v/v) acetonitrile/water with 0.1% (v/v) TFA in 60 minutes on a Waters Delta Pak C18-100A (3.9x150mm, 5mm) column at 1 ml/min.

Amino acid analysis. Amino acid analysis was performed using a Waters Pico-Tag system, after hydrolysis in a Pico-Tag workstation using 6N HCl at 150°C for 1 hour, and derivatization with phenylisothiocyanate.

Preparative HPLC. Preparative HPLC was carried out using a Waters Prep 4000 liquid chromatograph, equipped with a Waters RCM module with two PrepPak cartridges plus guard cartridge (40x210 mm or 25x210 mm) filled with Delta-Pak C18-100A (15 mm) material. Peptides were detected at 230 nm using a Waters 486 spectrophotometer with a preparative cell.

Molecular modeling. Molecular modeling was performed on a Silicon Graphics Iris Indigo Elan workstation (Silicon Graphics, Mountain View, CA, USA) using SYBYL molecular modeling software version 6.1 (Tripos Associates, St. Louis, MO, USA).

Energy minimizations were performed using the Sybyl 6.1 Tripos force field [Clark et al. Science 273:458-463 (1989)].

Multiple peptide synthesis. We used a Hamilton Microlab 2200 (Reno, NV, USA) to synthesize up to 40 peptides simultaneously at 30 mmol scale. The Hamilton Microlab 2200 was programmed to deliver washing solvents and reagents to two racks with 20 individual 4 ml columns with filter, containing resin for peptide synthesis. The columns were drained automatically after each step by vacuum. The coupling cycle was based on Fmoc/HBTU chemistry [Fields et al. Peptide Res. 4: 95-101 (1991)] using double coupling steps of 40 min. After coupling of the last amino acid, the Fmoc group was removed using 30% (v/v) piperidine/NMP for 3 and for 15 minutes. The peptides were washed with NMP (5 times), acetylated using NMP/acetic anhydride/DIEA (10/1/0.1; v/v/v) for 30 minutes, washed succesively with NMP and ethanol, and then dried. Peptides were deprotected and cleaved in 2 hr using 1.5 ml of a mixture of TFA/phenol/TA/water/EDT (10/0.75/0.5/0.5/0.25; v/w/v/v/v/) and then precipitated twice by adding hexane/diethylether (1/1; v/v). The precipitate was dried and lyophilized from water/acetonitrile (1/1; v/v).

Peptide cyclization. For air oxidation; peptides (MBG03 or MBG04) were dissolved at 0.5 mg/ml in 1% (w/v) ammonium bicarbonate/water and slowly stirred. At several intervals samples were taken for HPLC analysis and the amount of free thiol groups was measured in uv at 412 nm after reaction with 5,5'-dithiobis(2-nitrobenzoic acid) according to Ellman [Ellman Biochem. Biophys. 82: 70-77 (1959)]. For DMSO oxidation; peptides (MBG03 or MBG04) were dissolved at 0.5 mg/ml in 20% (v/v) DMSO/0.1 M phosphate buffer (pH 5). At several intervals samples were taken for HPLC analysis and for the Ellman test.

Antifungal activity. Antifungal activity assays were carried out in microtiter plates [Broekaert et al. FEMS Microbiol. Lett. 69: 55059 (1990)] with twofold serial dilutions of peptides and spore suspensions of the fungus *Fusarium culmorum* (IMI 180420) or other fungi. Growth of the fungus was monitored microscopically and spectrophotometrically after 72 hrs at room temperature in a medium of half-strength potato dextrose broth (1/2 PDB, Difco) at pH5.8. In some experiments, medium 1/2PDB was buffered by addition of 10mM MES at pH5.0 as indicated.

Alternatively, a synthetic medium was used consisting of the constituents of SMF (Cammue *et al.* J. Biol. Chem. 267; 2228-2233 (1992)] with addition of 1 mH $CaCl_2$, 50mM KCl and 10 mM Tris, and adjusted at pH 7.0. This medium is called SMF+pH7. Medium SMF+pH5 is identical to SMF+pH7 except that 10mM MES was added instead of 10mM Tris and that the pH was adjusted to 5.0. Results are expressed as concentration in μg/ml that gives 50% growth inhibition (IC50) as described by Cammue *et al* [J. Biol Chem. 267, 2228-2233 (1992)].

Antibacterial activity. Antibacterial activity was determined as described in Cammue *et al* [J. Biol. Chem. 267, 2228-2233 (1992)]. The medium consisted of either 1% trypton and 0.5% low melting point agarose (TA medium) or TA medium supplemented with 1mM $CaCl_2$ and 50mM KCl (TA+medium).

Results are expressed as concentration in μg/ml that gives 50% growth inhibition (IC_{50}).

Ca^{2+} influx assay. Neurospora crassa and *Fusarium culmorum* were grown at inoculum densities of $3x10^5$ and $5x10^4$ spores/ml respectively, in a 100 ml erlenmeyer flask placed on a rotary shaker in 1/2 PDB supplemented with 0.5 mCi [^{3}H]N-acetyl-D-glucosamine/ml [Thevissen ey al. J. Biol. Chem, 271: 15018-15025 (1996)]. After 20 h of incubation at 22°C, 2 mCi/ml $^{45}CaCl_2$ was added together with the peptides. After appropriate incubation times, 250 ml samples were taken and transferred to wells of a MultiScreen Durapore 96-well filtration plate (Millipore, Bedford, MA, USA), placed on a MultiScreen vacuum filtration manifold (Millipore). After filtration, harvested hyphae were washed four times with 250 ml of 10 mM $CaCl_2$. Membranes with hyphae were punched out manually with Multiscreen punch tips (Millipore), and counted for ^{3}H and ^{45}Ca in a Liquid Scintillation Counter (Wallac 1410, Pharmacia, Uppsala, Sweden). Background counts were negligible. In order to relate the ^{3}H counts to biomass, a 10 ml sample of the culture was filtered on a preweighted Millipore glass fibre filter, dried in a vacuum dessicator, weighted again and counted for ^{3}H.

RESULTS

It has previously been demonstrated that synthetic peptides corresponding to parts of the sequence of the plant defensin RsAFP2 show antifungal activity (De Samblanx et al. Peptide Research 1996, 9: 262 - 268 + Published International Patent Application No. WO 97/21815).

As these peptides invariably contain at least one cysteine residue, they can form intra - or intermolecular disulphide bridges due to air oxidation under testing conditions. This could result in the formation of a mixture of linear monomeric, cyclic monomeric and multimeric compounds making it difficult to understand which of these compounds contributes most to the antifungal activity. Two series of synthetic peptides were synthesised. A first series consisted of derivatives of the 6- mer peptide, called MCH 39, which corresponds to the sequence of RsAFP2 from position 43 to 48 encompassing two cysteines. The derivatives had either one or two of their cysteines replaced by alpha - aminobutyric acid, or had additional cysteines at the N-terminal end, the C-terminal end or both ends. After synthesis, the fractions containing either linear monomeric peptides, cyclic monomeric peptides or dimeric peptides were separated and tested for antifungal activity against *Fusarium culmorum* in three different media, ½ PDB, SMF + pH5 or SMF + pH7 (Table 1). The only peptides which was completely devoid of antifungal activity was the peptide MCH36 which had no cysteines. This indicates that cysteines are required for such small peptides to exert antifungal activity. In all cases fractions containing dimers (due to the formation of an intermolecular disulphide bride) had a stronger antifungal potency than the linear monomeric forms. Especially noteworthy is the strong antifungal potency of the compound found by self-dimerisation of the 6-mer peptide MCH38 containing a single cysteine.

A second set of derivatives was produced based on the 15-mer peptide MCI14, which corresponds to the RsAFP2 sequence from positions 33 to 47 encompassing three cysteines. Also in this case one or more of the cysteines were replaced by alpha-aminobutyric acid, or an extra cysteine was added at the N-terminal end, the C-terminal end or both ends. Data on the antifungal activity of this peptide series are shown in table 2 . Also in this series, the only peptide that had no antifungal activity was the one (MCI18) lacking any cysteines.

A 19-mer peptide (MBG01), whose sequence corresponded to the Rs-AFP2 sequence between Ala-31 and Phe-49 was synthesized. This sequence was selected such that it emcompassed the beta-2strand/beta turn/beta 3-strand region of RsAFP2. The concentration of MBG01 required for 50% growth inhibition of the fungus *Fusarium*

culmorum (IC_{50}) was 33 ug/ml, which is about seven fold higher than the IC_{50} value of Rs-AFP2 itself (Table 3).

As MBG01 contains three cysteine residues, it can form various intra- and intermolecular disulfide bridges, due to air-oxidation under testing conditions. It is therefore likely that MBG01 exists as a mixture of various multimers and cyclic monomers and it is difficult to assess which form is actually responsible for the antifungal effects. In order to avoid such problems with uncontrolled cystine formation, an analogue of MBG01 was synthesized (MBG02) in which all three cysteine residues had been substituted by alpha aminobutyric acid. Surprisingly, MBG02 was even more potent than MBG01 and showed an IC_{50} value of 8 ug/ml (Table 3).

Two further analogues of MBG01 were synthesized both of which were forced to adopt a more rigid loop-like structure. Peptide MBG03 is identical to MBG02 (including the three alpha-aminobutyric acid residues) except that the terminal amino acid residues alanine and phenylalanine were replaced by cysteines. Peptide MBG04 is identical to MBG03, except for a substitution of the the alpha-aminobutyric acid residues at positions 36 and 45 by two D-cysteines. Energy minimization calculations showed that a cystine bridge can be formed between the two cysteines of MBG03 while the peptide may adopt a conformation close to that of the corresponding loop in Rs-AFP2. Likewise, it was shown by molecular modeling that two cystine bridges can be formed between the two L-cysteines and two D-cysteines respectively, of MBG04 and that the resulting conformation is energetically feasible. Cystine bridge formation in MBG03 was performed under two different conditions: air oxidation at pH 8 and DMSO oxidation at pH 5-6. Both oxidations were completed within 40 hrs according to Ellman's test [Ellman Biochem. Biophys. 82: 70-77 (1959)]. HPLC analysis showed that air oxidation at pH 8 allowed rearrangements to unwanted multimeric products, however, DMSO oxidation resulted in one main product. The oxidation of MBG04, introducing two cystine bridges, was performed under similar conditions as MBG03. Again, air oxidation at pH 8 resulted in a complex mixture of unwanted products, while DMSO oxidation at pH 5-6 gave one main product. MBG03 and MBG04 were purified using preparative HPLC and tested for antifungal potency (Table 3). MBG03 and MBG04 were both found to be active against *F. culmorum*, with IC_{50} values of 17 and 15 ug/ml, respectively.

Rs-AFP2 has previously been shown to mediate Ca^{2+}-influx into fungal hyphae treated with this protein [Thevissen et al. J. Biol. Chem, 271: 15018-15025 (1996)]. Therefore, Rs-AFP2 and the synthetic 19-mer peptides MBG01 and MBG02 were tested alongside in a Ca^{2+}-influx assay on pregerminated *F.culmorum* hyphae. MAT02 (Table 3), a peptide corresponding to the Rs-AFP2 sequence from Gln-5 till Glu-29 and which was previously found to be devoid of antifungal activity was included as a negative control. MAT02 was found to be inactive in the Ca^{2+}-influx assay. In contrast, MBG01, MBG02 and Rs-AFP2 caused a dramatically increased Ca^{2+} influx from 0.3 mM and upwards. The doses for half maximal effect were around 2-4 mM for MBG01, MBG02 and Rs-AFP2.

In earlier work, using PEPSCAN analysis, we found that the activity of Rs-AFP2 was mainly located in the loop sequence consisting of the beta2 strand, the beta turn around Pro-41, and the antiparallel beta3 strand [De Samblanx et al Peptide Res. 9 (1996) 262-268]. In this work we used molecular modeling based on NMR-derived 3D structure data of Rs-AFP1 [Fant et al. J. Mol. Biol. 279, 257-270 (1998)] to define the optimal length of the loop sequence. Based on these results, we synthesized cyclic peptides with increasing rigidity by the introduction of one or two cysteine bridges and tested them for antifungal potency.

Because the three cysteines in the loop sequence were not needed for biological activity, we used two of them and the terminal amino acid residues to make the loop structure more rigid. We substituted Ala-31 and Phe-49 by cysteines to build a single intramolecular disulphide bridge (MBG03) or, in addition, Cys-36 and Cys-45 by D-cysteines to form two intramolecular disulphide bridges (MBG04). D-cysteines were chosen because the side-chain sulphydryl groups in the model were located closer to each other compared with L-cysteines and only very small effects on the structure were found after energy minimalization of the bicyclic compound. The antifungal activity of both cyclic peptides was similar to that of the linear peptides. This means, that the conformation of all four loop peptides closely resembles the native structure or is still flexible enough, even with two disulfide bridges, to exert its activity on the fungal hyphae.

TABLE 1 Results of synthesis and antifungal activity of loop-constructs of Rs-AFP2[a]. Rs-AFP active 6-mer based peptides (region 42-48) in media 1/2PDB, SMF=pH5 (both 10 mM MES buffered) and SMF+pH7 (10 mM Tris/HCl buffered)

Code	Peptide sequence		SEQ ID NO	IC50 value (µg/ml) in medium: ½ PDB	SMF + pH5	SMF + pH7
	OD100% :			0.346	0.079	0.059
MCH32	*HKBIBYC#	lin f1	4	74.8	>400	>400
MCH32x	*HKBIBYC#	dim f4	4	27.2	155.2	>400
MCH33	*CHKBIBY#	lin f1	5	129.7	>400	>400
MCH33x	*CHKBIBY#	dim f1	5	71.4	>400	>400
MCH36	*HKBIBY#	lin f1	6	>400	>400	>400
MCH37	*HKCIBY#	lin f5	7	44.3	>400	>400
MCH37x	*HKCIBY#	dim f2	7	17.9/50.5	>400	>400
MCH38	*HKBICY#	lin f1	8	<12.5/16.3	>400	268.1
MCH38x	*HKBICY#	dim f1	8	<12.5/8.3	81.8	<12.5/12.2
MCH39	*HKCICY#	lin f1	9	16.9	374.1	354.2
MCH39x	*HKCICY#	ox f1	9	<12.5/5.6	176.3	42.0
MCI28	*CHKBIBYC#	lin f2	10	37.3	>400	>400
MCI28x	*CHKBIBYC#	cyc f2	10	109.6	>400	>400

Peptides were tested on spores of *Fusarium culmorum* ($2x10^4$ spores/ml); mean of duplo experiments (n=2) after 72 hour of incubation. Optical density of 100%-growth control wells (no peptide added) is indicated. *=acetyl, # = amide, B=alpha-amino butyric acid; lin=linear; dim=dimer; cyc=cyclic; ox=undefined oxidized product; f=fraction number

TABLE 2

Rs-AFP β2-β3 15- to 17-mer loop peptides in media 1/2PDB, SMF+pH5 (both 10 mM MES buffered) and SMF+pH7(10 mM Tris/HCl buffered)

Code	Peptide sequence		SEQ ID NO	IC50 value (μg/ml) in medium:		
				½ PDB	SMF+pH5	SMF+pH7
	OD100%:			0.346	0.079	0.059
MCI10	*CHGSBNYVFPAHKBIBC#	linf1	11	40.5	>400	>400
MCI10x	*CHGSBNYVFPAHKBIBC#	cycf1	11	54.8	>400	>400
MCI11	*CHGSBNYVFPAHKBIB#	lin f1	12	83.2	>400	>400
MCI11x	*CHGSBNYVFPAHKBIB#	dim f3	12	51.1	>400	>400
MCI12	*HGSBNYVFPAHKBIBC#	lin f1	13	65.7	>400	>400
MCI12x	*HGSBNYVFPAHKBIBC#	dim f2	13	24.5	>400	>400
MCI14	*HGSCNYVFPAHKCIC#	lin f1	14	<12.5/9.6	>400	387.7
MCI14x	*HGSCNYVFPAHKCIC#	ox f1	14	<12.5/4.1	>400	>400
MCI15	*HGSCNYVFPAHKBIB#	lin f1	15	79.4	>400	>400
MCI15x	*HGSCNYVFPAHKBIB#	dim f3	15	59.3	>400	>400
MCI16	*HGSBNYVFPAHKCIB#	lin f1	16	14.6/<12.5	>400	>400
MCI16x	*HGSBNYVFPAHKCIB#	dim f2	16	<12.5/4.9	>400	>400
MCI17	*HGSBNYVFPAHKBIC#	lin f2	17	17.9	>400	>400
MCI17x	*HGSBNYVFPAHKBIC#	dim f3	17	<12.5/14.8	>400	>400
MCI18	*HGSBNYVFPAHKBIB#	lin f3	18	>400	>400	>400

Peptides were tested on spores of *Fusarium culmorum* ($2x10^4$ spores/ml); mean of duplo experiments (n=2) after 72 hour of incubation. Optical density of 100%-growth control wells is indicated. *=acetyl, # = amide, B=alpha-amino butyric acid; lin=linear; dim=dimer; cyc=cyclic; ox=undefined oxidized product; f=fraction number

TABLE 3

Table 3. Results of synthesis and antifungal activity of loop-constructs of Rs-AFP2 [a].

Code	sequence[b]	SEQ ID NO	yield(mg)	purity[c]	IC_{50}[d]	IC_{50}[f]
MBG01	*ARHGSCNYVFPAHKCICYF#	19	59.8	80	33	50
MBG02	*ARHGSBNYVFPAHKBIBYF#	20	57.8	90	8	22
MBG03	*CRHGSBNYVFPAHKBIBYC#	21	51.0	80	17[e]	36
MBG04	*CRHGScNYVFPAHKcIBYC#	22	61.0	80	15[e]	nd
MBG06	*ARHGSC#	23	19.7	80	159	nd
MBG08	*ARHGSB#	24	22.7	90	>400	nd
MBN06	*CRHGSCNYVFPAHKCIBYC#	25	69.0	70	39	nd
MAT 02	*QRPSGTWSGVCGNNN#	26	14.1	40	>400	nd

[a] Multiple peptide synthesis at 15 (MAT02) or 30 mmol scale [b] *=acetyl, # = amide, B=alpha aminobutyric acid, c = D-Cys. [C] % of peak area as estimated from HPLC at 215nm. [d] concentration in ug/ml that gives 50% growth inhibition of Fusarium culmorum in non-buffered medium. [e] cyclised and purified product [f] in 10mM MES buffered medium nd = not determined

CLAIMS

1. A modified cysteine-containing antimicrobial peptide derived from a plant defensin said modification comprising (a) introducing one or more cysteine residues and/or (b) replacing or altering one or more, cysteine residues to block their ability to form disulphide bridges.

2. A modified cysteine-containing antimicrobial peptide according to claim 1 which is derived from a naturally-occurring plant defensin.

3. A modified cysteine containing antimicrobial peptide according to claim 1 or claim 2 wherein said peptide is derived from the beta-2 strand/turn/beta-3 strand region of a plant defensin.

4. A modified cysteine containing antimicrobial peptide according to any one of the preceding claims which are derived from plant defensins having substantially similar activity to Rs-AFP2, and which show at least 40%, sequence similarity to Rs-AFP2.

5. A modified cysteine containing antimicrobial peptide according to claim 4 which are derived from Rs-AFP1, Rs-AFP3, Rs-AFP4, Br-AFP1, Br-AFP2, Bn-AFP1, Bn-AFP2, Sa-AFP1, Sa-AFP2 and At-AFP1 and Hs-AFP2, Ah-AMP1 or Dm-AMP1

6. A modified cysteine containing antimicrobial peptide according claim 5 which is derived from Rs-AFP2 or Rs-AFP1

7. A modified cysteine containing antimicrobial peptide according to claim 6 derived from positions 21 to 51 of Rs-AFP1 or Rs-AFP2 .

8. A modified cysteine containing antimicrobial peptide according to claim 7 wherein said peptide is derived from position 21 to 51 of the Rs-AFP2 sequence.

9. A modified cysteine containing antimicrobial peptide according to any of the preceding claims wherein the one or more cysteines are added to the N-terminal and/or C-terminal ends of the peptide.

10. A modified cysteine containing antimicrobial peptide according to claim 1 and selected from MBG03 CRHGSBNYVFPAHKBIBYC (SEQ ID NO 21), MBG04 CRHGScNYVFPAHKcIBYC (SEQ ID NO 22) , MBN06 CRHGSCNYVFPAHKCIBYC (SEQ ID NO 25), MCH32 and MCH32x HKBIBYC (SEQ ID NO 4), MCH33 and MCH33x CHKBIBY (SEQ ID NO 5), MCH37 and MCH37x HKCIBY (SEQ ID NO 7), MCH38 and MCH38x HKBICY (SEQ ID NO 8), MCI28 and MCI28x CHKBIBYC (SEQ ID NO 10), MCI10 and MCI10x CHGSBNYVFPAHKBIBC (SEQ ID NO 11), MCI11 and MCI11x CHGSBNYVFPAHKBIB (SEQ ID NO 12), MCI12 and MCI12x HGSBNYVFPAHKBIBC (SEQ ID NO 13), MCI15 and MCI15x HGSCNYVFPAHKBIB (SEQ ID NO 15), MCI16 and MCI16x HGSBNYVFPAHKCIB (SEQ ID NO 16) and MCI17 and MCI17x HGSBNYVFPAHKBIC (SEQ ID NO 17),.

11. An antimicrobial peptide comprising a plant defensin or a peptide derivative thereof, comprising one or more cysteine residues in the D-configuration.

12. A method of making antimicrobial peptides more rigid comprising modifying the peptide structure by (a) introducing one or more cysteine residues and/or (b) replacing or altering one or more cysteine residues to block their ability to form disulphide bridges.

13. A method according to claim 12 wherein the antimicrobial peptide is made more rigid by altering one or more, but not all, cysteine residues to block their ability to form disulphide bridges.

14. A method of improving the antimicrobial activity of an antimicrobial peptide derived from a plant defensin said method comprising altering the natural disulphide bridge pattern of the peptide by (a) introducing one or more cysteine residues and/or (b) replacing or altering or one or more, but not all cysteine residues to block their ability to form disulphide bridges

15. A process of combating fungi whereby they are exposed to an antimicrobial peptide according to any one of claims 1 to 10.

16. A composition comprising an antimicrobial peptide according to any one of claims 1 to 10.

17. A plant having improved resistance to a fungal or microbial pathogen and containing recombinant DNA which expresses an antimicrobial peptide according to any of claims 1 to 10, provided said peptide contains unaltered amino acids.

18. Use of a peptide according to any of claims 1 to 10 in the treatment or prevention of microbial infections.

19. Use according to claim 18 wherein the microbial infection is a fungal infection.

20. A DNA sequence encoding an antimicrobial peptide according to any one of claims 1 to 10.

21. A vector comprising a DNA sequence according to claim 17.

SEQUENCE LISTING

<110> Zeneca Limited
Rees, Sarah B
Osborn, Rupert W
Posthuma, Geertruida A
Schaaper, Wilhelmus MM
Sijtsma, Lolke
Amerongen, Aart V
Fant, Franky
Borremans, Frans AM

<120> Synthetic peptides

<130> PPD 50286/WO

<140>
<141>

<150> GB 9918156.2
<151> 1999-08-02

<160> 26

<170> PatentIn Ver. 2.1

<210> 1
<211> 51
<212> PRT
<213> Artificial Sequence

<220>
<221> VARIANT
<222> (1)..(3)

<220>
<221> VARIANT
<222> (5)..(14)

<220>
<221> VARIANT
<222> (26)..(35)

<220>
<221> VARIANT
<222> (37)..(44)

<220>
<221> DISULFID
<222> (4)..(51)

<220>
<221> DISULFID
<222> (15)..(36)

<220>
<221> DISULFID
<222> (21)..(45)

<220>
<221> DISULFID
<222> (25)..(47)

<220>
<221> SITE
<222> (11)
<223> Xaa = aromatic amino acid (Phe, Trp, Tyr)

<220>
<221> SITE
<222> 1..3, 5..10, 12, 14, 16..20, 22..24, 26..28
<223> Xaa = any amino acid

<220>
<221> SITE
<222> 30..33, 35, 37..44, 46, 48..50
<223> Xaa = any amino acid

<220>
<221> SITE
<222> (11)
<223> Xaa is an aromatic amino acid (Phe, Trp, Tyr)

<220>
<223> Description of Artificial Sequence: Common
structural characteristic of plant defensins

<400> 1
Xaa Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Gly Xaa Cys Xaa
1 5 10 15

Xaa Xaa Xaa Xaa Cys Xaa Xaa Xaa Cys Xaa Xaa Xaa Glu Xaa Xaa Xaa
20 25 30

Xaa Gly Xaa Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Cys Xaa
35 40 45

Xaa Xaa Cys
50

<210> 2
<211> 51
<212> PRT
<213> Raphanus sativus

<400> 2
Gln Lys Leu Cys Gln Arg Pro Ser Gly Thr Trp Ser Gly Val Cys Gly
1 5 10 15

Asn Asn Asn Ala Cys Lys Asn Gln Cys Ile Arg Leu Glu Lys Ala Arg
20 25 30

His Gly Ser Cys Asn Tyr Val Phe Pro Ala His Lys Cys Ile Cys Tyr
35 40 45

Phe Pro Cys
50

<210> 3
<211> 51
<212> PRT
<213> Raphanus sativus

<400> 3
Gln Lys Leu Cys Glu Arg Pro Ser Gly Thr Trp Ser Gly Val Cys Gly
1 5 10 15

Asn Asn Asn Ala Cys Lys Asn Gln Cys Ile Asn Leu Glu Lys Ala Arg
20 25 30

His Gly Ser Cys Asn Tyr Val Phe Pro Ala His Lys Cys Ile Cys Tyr
35 40 45

Phe Pro Cys
50

<210> 4
<211> 7
<212> PRT
<213> Artificial Sequence

<220>
<221> MOD_RES
<222> (3, 5)
<223> Abu

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 4
His Lys Xaa Ile Xaa Tyr Cys
1 5

<210> 5
<211> 7
<212> PRT
<213> Artificial Sequence

<220>
<221> MOD_RES
<222> (4, 6)
<223> Abu

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 5
Cys His Lys Xaa Ile Xaa Tyr
1 5

<210> 6
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<221> MOD_RES
<222> (3, 5)
<223> Abu

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 6
His Lys Xaa Ile Xaa Tyr
1 5

<210> 7
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<221> MOD_RES
<222> (5)
<223> Abu

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 7
His Lys Cys Ile Xaa Tyr
1 5

<210> 8
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<221> MOD_RES
<222> (3)
<223> Abu

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 8
His Lys Xaa Ile Cys Tyr
1 5

<210> 9
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 9
His Lys Cys Ile Cys Tyr
1 5

<210> 10
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<221> MOD_RES
<222> (4, 6)
<223> Abu

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 10
Cys His Lys Xaa Ile Xaa Tyr Cys
1 5

<210> 11
<211> 17
<212> PRT
<213> Artificial Sequence

<220>
<221> MOD_RES
<222> (5, 14, 16)
<223> Abu

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 11
Cys His Gly Ser Xaa Asn Tyr Val Phe Pro Ala His Lys Xaa Ile Xaa
1 5 10 15

Cys

<210> 12
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
<221> MOD_RES
<222> (5, 14, 16)
<223> Abu

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 12
Cys His Gly Ser Xaa Asn Tyr Val Phe Pro Ala His Lys Xaa Ile Xaa
1 5 10 15

<210> 13
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
<221> MOD_RES
<222> (4, 13, 15)
<223> Abu

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 13
His Gly Ser Xaa Asn Tyr Val Phe Pro Ala His Lys Xaa Ile Xaa Cys
1 5 10 15

<210> 14
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 14
His Gly Ser Cys Asn Tyr Val Phe Pro Ala His Lys Cys Ile Cys
1 5 10 15

<210> 15
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<221> MOD_RES

<222> (13, 15)
<223> Abu

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 15
His Gly Ser Cys Asn Tyr Val Phe Pro Ala His Lys Xaa Ile Xaa
1 5 10 15

<210> 16
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<221> MOD_RES
<222> (4, 15)
<223> Abu

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 16
His Gly Ser Xaa Asn Tyr Val Phe Pro Ala His Lys Cys Ile Xaa
1 5 10 15

<210> 17
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<221> MOD_RES
<222> (4, 13)
<223> Abu

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 17
His Gly Ser Xaa Asn Tyr Val Phe Pro Ala His Lys Xaa Ile Cys
1 5 10 15

<210> 18
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<221> MOD_RES
<222> (4, 13, 15)
<223> Abu

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 18
His Gly Ser Xaa Asn Tyr Val Phe Pro Ala His Lys Xaa Ile Xaa
1 5 10 15

<210> 19
<211> 19
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 19
Ala Arg His Gly Ser Cys Asn Tyr Val Phe Pro Ala His Lys Cys Ile
1 5 10 15

Cys Tyr Phe

<210> 20
<211> 19
<212> PRT
<213> Artificial Sequence

<220>
<221> MOD_RES
<222> (6, 15, 17)
<223> Abu

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 20
Ala Arg His Gly Ser Xaa Asn Tyr Val Phe Pro Ala His Lys Xaa Ile
1 5 10 15

Xaa Tyr Phe

<210> 21
<211> 19
<212> PRT
<213> Artificial Sequence

<220>
<221> MOD_RES
<222> (6, 15, 17)
<223> Abu

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 21
Cys Arg His Gly Ser Xaa Asn Tyr Val Phe Pro Ala His Lys Xaa Ile
1 5 10 15

Xaa Tyr Cys

<210> 22
<211> 19
<212> PRT
<213> Artificial Sequence

<220>
<221> SITE
<222> (6, 15)
<223> Xaa = D-Cys

<220>
<221> MOD_RES
<222> (17)
<223> Abu

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 22
Cys Arg His Gly Ser Xaa Asn Tyr Val Phe Pro Ala His Lys Xaa Ile
1 5 10 15

Xaa Tyr Cys

<210> 23
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 23
Ala Arg His Gly Ser Cys
1 5

<210> 24
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<221> MOD_RES
<222> (6)
<223> Abu

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 24
Ala Arg His Gly Ser Xaa
1 5

<210> 25
<211> 19
<212> PRT
<213> Artificial Sequence

<220>
<221> MOD_RES
<222> (17)
<223> Abu

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 25
Cys Arg His Gly Ser Cys Asn Tyr Val Phe Pro Ala His Lys Cys Ile
1 5 10 15

Xaa Tyr Cys

<210> 26
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 26
Gln Arg Pro Ser Gly Thr Trp Ser Gly Val Cys Gly Asn Asn Asn
1 5 10 15